ANIMAL RESEARCH PAPER

Comparison of fermentation characteristics and bacterial diversity in the rumen of sheep and in batch cultures of rumen microorganisms

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SUMMARY

The objective of the current study was to assess how closely batch cultures (BC) of rumen microorganisms can mimic the dietary differences in fermentation characteristics found in the rumen, and to analyse changes in bacterial diversity over the *in vitro* incubation period. Four ruminally and duodenally cannulated sheep were fed four diets having forage : concentrate ratios (FCR) of 70 : 30 or 30 : 70, with either alfalfa hay or grass hay as forage. Rumen fluid from each sheep was used to inoculate BC containing the same diet fed to the donor sheep, and the main rumen fermentation parameters were determined after 24 h of incubation. There were differences between BC and sheep in the magnitude of most measured parameters, but BC detected differences among diets due to forage type similar to those found in sheep. In contrast, BC did not reproduce the dietary differences due to FCR found in sheep for pH, degradability of neutral detergent fibre and total volatile fatty acid (VFA) concentrations. There were differences between systems in the magnitude of most determined parameters and BC showed higher pH values and NH_3–N concentrations, but lower fibre degradability and VFA and lactate concentrations compared with sheep. There were significant relationships between *in vivo* and *in vitro* values for molar proportions of acetate, propionate and butyrate, and the acetate : propionate ratio. The automated ribosomal intergenic spacer analysis (ARISA) of 16S ribosomal deoxyribonucleic acid showed that FCR had no effect on bacterial diversity either in the sheep rumen fluid used as inoculum (IN) or in BC samples. In contrast, bacterial diversity was greater with alfalfa hay diets than those with grass hay in the IN, but was unaffected by forage type in the BC. Similarity index between the bacterial communities in the inocula and those in the BC ranged from 67·2 to 74·7%, and was unaffected by diet characteristics. Bacterial diversity was lower in BC than in the inocula with 14 peaks out of a total of 181 detected in the ARISA electropherograms never appearing in BC samples, which suggests that incubation conditions in the BC may have caused a selection of some bacterial strains. However, each BC sample showed the highest similarity index with its corresponding rumen IN, which highlights the importance of using rumen fluid from donors fed a diet similar to that being incubated in BC when conducting *in vitro* experiments.

INTRODUCTION

The complexity of the rumen ecosystem, the difficulties of working with fistulated animals and the increased public awareness of animal rights have contributed to the development of numerous *in vitro* techniques to simulate rumen fermentation. In recent years, there has been an increased use of batch cultures (BC) of ruminal microorganisms, which are being widely utilized for nutritive evaluation and screening feed additives, among other multiple purposes. The use of BC is a rapid and precise *in vitro* method that needs only a small amount of substrate, but requires an inoculum (IN) which may influence the fermentation process (Mould *et al.* 2005). As has been previously stated (Warner 1956; Czerkawski &

Breckenridge 1977), it is challenging to evaluate how closely an *in vitro* system can mimic the *in vivo* rumen and to select the most appropriate parameters for measurement. To date, studies comparing fermentation patterns in BC with those found *in vivo* are very limited and have contradictory results. Rymer & Givens (2002) tested three mixed diets and found significant relationships between the volatile fatty acid (VFA) pattern produced in BC and in the rumen of sheep. In contrast, Brown *et al.* (2002) found no relationship between the VFA pattern measured in BC and in the rumen of steers fed eight different forages, and attributed the lack of correlation between *in vitro* and *in vivo* data to differences in the diets fed to the steers and those fed to the ruminal fluid donors in the *in vitro* trial.

Any *in vitro* system designed to simulate rumen fermentation should mimic the natural rumen, including the physical environment and the maintenance of key microbial populations (Ziemer *et al.* 2000). Ideally, the microbial composition in the *in vitro* system should be representative, in terms of quantity and quality, of that found in the rumen of the host animal. Despite the importance of this aspect, the number of studies comparing microbial populations in the rumen with those in the *in vitro* systems is limited, and most of them have been conducted in continuous fermenters. To our knowledge, only Prates *et al.* (2010) have compared the structure of bacterial communities in BC with that in the rumen fluid used as IN, and reported a mean similarity index of 70·2% between both bacterial communities; this would indicate that some changes in bacterial composition were produced over the 24 h incubation period, although only one diet was tested in that study and the magnitude of the changes may be influenced by the composition of the incubated diet.

The objectives of the current study were to assess how closely BC can mimic the *in vivo* rumen fermentation of diets of variable composition and to analyse changes in bacterial communities over the incubation period as assessed by automated ribosomal intergenic spacer analysis (ARISA). For that purpose, four different diets were incubated in BC, and the results were validated with those determined in sheep fed the same diets.

MATERIALS AND METHODS

Animals, diets and experimental design

All the experimental procedures were approved by the León University Institutional Animal Care and Use Committee. The *in vitro* experiment was conducted in conjunction with an *in vivo* study analysing the effects of forage-to-concentrate ratio (FCR) and type of forage on rumen fermentation characteristics, diet digestibility and microbial protein synthesis in sheep (Ramos *et al.* 2009*a*). For the current study, four of the six Merino sheep utilized in the previous study were selected. The sheep (58·5 ± 3·16 kg of body weight) were fitted with permanent ruminal and T-type duodenal cannulae. Animals were housed in individual pens and had continuous access to fresh water and vitamin–mineral blocks over the experimental period.

The four experimental diets had high forage (HF; 700 g/kg; dry matter (DM) basis) or high concentrate (HC; 700 g/kg DM) content, with either alfalfa hay (HFA and HCA) or grass hay (HFG and HCG) as forage. The same concentrate was used in all the diets. Ingredient and chemical composition of diets are shown in Table 1. Diets were fed twice daily (08·00 and 20·00 h) at a daily rate of 56 g DM/metabolic weight (kg body weight$^{0·75}$) to minimize feed selection. Diet intake was monitored daily and all animals consumed all the diet offered, with the exception of one sheep that occasionally left some refusals (<100 g/day) of diet HFG.

The experiment had a 4 × 4 Latin square design with four 34-day experimental periods. Each period consisted of 22 days of dietary adaptation, 7 days (days 23–30) for determination of *in vivo* parameters, and 2 days for conducting the *in vitro* experiment. Sheep data collection included measurements of rumen fermentation characteristics (pH and concentrations of ammonia (NH_3–N), VFA and lactate) and of rumen apparent degradability of dry matter (DMD) and neutral detergent fibre (NDFD). On day 30 of each period, ruminal content samples (*c.* 50 g) were taken through the cannula of each sheep at 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 and 22 h after the morning feeding and strained through four layers of cheesecloth. The pH of the fluid was immediately measured, and three samples were taken: 5 ml of fluid were added to 5 ml of deproteinizing solution (100 g of metaphosphoric acid and 0·6 g of crotonic acid/litre) for VFA analysis, 2 ml were added to 2 ml of 0·5 M chloridric acid (HCl) for NH_3–N determination and 5 ml were frozen at −20 °C for total lactate analysis. In order to assess the duodenal flow of digesta, chromium-mordanted (Cr-mordanted) fibre and cobalt-ethylendiaminetetraacetic acid (Co-EDTA) were used as solid and fluid phase markers, respectively. From

Table 1. *Ingredient (g/kg DM) and chemical composition (g/kg DM) of the experimental diets with 700 g of forage/kg DM (HF) or 300 g of forage/kg DM (HC) and alfalfa hay (A) or grass hay (G) as forage*

Item	HFA	HFG	HCA	HCG
Ingredient				
Alfalfa hay	700	–	300	–
Grass hay	–	700	–	300
Barley	64	64	152	152
Gluten feed	61	61	145	145
Wheat middlings	57	57	137	137
Soybean meal	41	41	97	97
Palmkern meal	38	38	90	90
Wheat	15	15	35	35
Maize	15	15	35	35
Vitamin–mineral premix*	10	10	10	10
Chemical composition				
DM (g/kg fresh matter)	927	925	925	924
Organic matter	913	927	913	919
Nitrogen	26·9	19·4	28·3	25·6
NDF	426	499	374	401
ADF	269	238	187	174

* Vitamin–mineral premix contained per kilogram of DM: 11250 IU of vitamin A; 2250 IU of vitamin D_3; 25 mg of vitamin E; and 10 mg of $CuSO_45H_2O$.

days 18 to 26, 15 g of Cr-mordanted fibre were administered daily via the ruminal cannula into four equal portions at 08·00, 14·00, 20·00 and 02·00 h and Co-EDTA was infused into the rumen (60 mg of cobalt (Co) in 250 ml of distilled water/day) by means of a peristaltic pump. On days 23, 24 and 25 duodenal digesta samples were collected at 6 h intervals and samples were pooled by sheep and stored at −20 °C. Duodenal samples were thawed at 4 °C, homogenized, and one-half of each sample was centrifuged (1000 g, 5 min, 4 °C) to obtain particulate matter (Faichney 1975). Both samples of whole duodenal digesta and particulate matter were lyophilized and analysed for ash and neutral detergent fibre (NDF). More details on *in vivo* procedures are given in Ramos *et al.* (2009*a*).

Samples of each diet offered to sheep were ground (1 mm sieve) and used as substrates for the BC incubations. On day 32 of each experimental period, 400 g of rumen contents were taken through the cannula of each sheep immediately before the morning feeding and strained through four layers of cheesecloth. Rumen fluid from each sheep was mixed with the buffer–mineral solution of Goering & Van Soest (1970), modified to reduce the content of sodium bicarbonate (NH_4HCO_3) to half of its initial concentration with no trypicase added, in proportions of 1 : 4 (vol/vol) at 39 °C under continuous flushing with carbon dioxide (CO_2). About 100 ml of each mixture (IN) were placed immediately into sterile containers, frozen at −80 °C and freeze-dried before deoxyribonucleic acid (DNA) extraction. Four bottles (120 ml of volume) containing 400 mg DM of each diet were filled (40 ml/bottle) with the IN from the sheep fed the same diet. Bottles were capped and incubated at 39 °C. After 24 h, two bottles of each diet were opened, the pH was measured immediately and samples of the bottles' contents were taken for VFA, NH_3–N and total lactate analyses as described above. The contents of the bottles were then transferred to previously weighed filter crucibles, washed with 50 ml of hot (50 °C) distilled water, and the crucibles dried at 50 °C and weighed to calculate apparent DMD. Finally, the residue of the crucibles was analysed for NDF to calculate NDFD. The remaining two bottles of each diet were emptied into sterile containers, mixed, frozen immediately at −80 °C and freeze-dried before DNA extraction.

Deoxyribonucleic acid extraction and automated ribosomal intergenic spacer analysis

In order to analyse the changes in bacterial communities over the 24 h of incubation in the BC, DNA

was isolated in duplicate from samples (100 mg of DM) of IN and BC contents after mechanical disruption of microorganisms with a MiniBeadbeater (3 min; Biospec Products, Bartlesville, OK, USA). The DNA was extracted following the procedure described by Yu & Morrison (2004), with the exception that an additional step involving the treatment of samples with cetyltrimethylammonium bromide was included to remove polymerase chain reaction inhibitors. QIAamp DNA Stool Mini Kit columns (QIAgen, Valencia, CA, USA) were used to purify the DNA. Absorbance ratios (A260 : A280) of eluted DNA measured in a Nanodrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) were between 1·71 and 2·00. The DNA was amplified using universal bacterial primers 16S-1392F and 23S-125R as described by Saro *et al.* (2012). Thermocycling and the ARISA technique were conducted in a 2720 Thermal Cycler (Applied Biosystem, Foster City, CA, USA) and a MegaBACE 500 (Amersham Biosciences, Little Chalfont, Bucks, UK), respectively, as reported by Ramos *et al.* (2009*b*). Peaks were identified by comparison with an internal size standard using the GeneMarker Software v1·80 (SoftGenetics, State College, PA, USA) and the presence/absence of the different peaks was considered to compare the electropherogram profiles using a similarity matrix. Shannon's diversity index was used to evaluate the diversity of bacterial communities, and dendrograms were constructed using the per cent similarity and unweighted pair-group method using arithmetic averages options in the MVSP v3·12d software (Kovach Computing Service, Anglesey, Wales, UK).

Chemical analyses

Dry matter (ID 934·01), ash (ID 942·05) and nitrogen (ID 984·13) were determined according to the Association of Official Analytical Chemists (1999), and NDF and acid-detergent fibre (ADF) analyses were carried out according to Van Soest *et al.* (1991) in an ANKOM220 Fibre Analyser unit (ANKOM Technology Corporation, Fairport, NY, USA) using sodium sulphite and a heat-stable amylase. The concentration of ammonia nitrogen (NH_3–N) was determined using a spectrophotometer by the phenol–hypochlorite method (Weatherburn 1967) and those of VFA by gas chromatography as described by Carro *et al.* (1992). Analysis of lactate concentration was carried out following the procedure of Taylor (1996).

Calculations and statistical analyses

Duodenal flow was calculated from the concentrations of Cr and Co in duodenal digesta phases using the dualphase maker method of Faichney (1975). Samples were mathematically reconstituted to create a representative sample from the concentrations of each nutrient analysed in each of the duodenal phases (Faichney 1975). The concentrations of VFA, NH_3–N and lactate in sheep rumen were averaged across sampling times before statistical analysis (four values per diet). Values measured in the two bottles incubated for each IN in each period in the *in vitro* trial were averaged before statistical analysis (four values per diet).

In vivo and *in vitro* data were analysed separately to assess the effects of dietary treatments on each fermentation system. Data were analysed as a mixed model using the PROC MIXED of SAS (2012). The effects FCR, forage, FCR × forage interaction and period were considered fixed, while sheep (*in vivo* trial) and IN (*in vitro* study) effects were considered random. Effects were considered significant at $P \leq 0{\cdot}05$. Relationships between fermentation variables determined in BC and in sheep were tested by correlation analyses using the CORR procedure of SAS (2012).

RESULTS

The effects of FCR and type of forage on ruminal fermentation parameters and diet degradability in sheep and BC are shown in Table 2. There were no FCR × forage interactions for any measured variable in BC, with the exception of the molar proportion of valerate ($P = 0{\cdot}041$) and a trend for apparent DMD ($P = 0{\cdot}060$). This agrees well with the results obtained in sheep, in which FCR × forage interaction was only found for valerate proportion ($P < 0{\cdot}001$) and a trend for apparent DMD ($P = 0{\cdot}079$).

Final pH values in BC were 6·76, 6·83, 6·65 and 6·69 for diets HFA, HFG, HCA and HCG, respectively, whereas mean pH values in sheep rumen were 6·36, 6·49, 6·23 and 6·17, respectively. Reducing the forage proportion in the diet decreased rumen pH in sheep ($P = 0{\cdot}003$), but only a trend was observed in the BC ($P = 0{\cdot}064$). While fermentation of HF diets in sheep resulted in greater ($P < 0{\cdot}05$) apparent DMD and NDFD compared with HC diets, HF diets in BC tended to have lower ($P = 0{\cdot}052$) DMD than HC diets and no differences ($P = 0{\cdot}222$) in NDFD were observed. Concentrations of NH_3–N were

Table 2. *Effect of forage : concentrate ratio (FCR) and type of forage on apparent degradability(g/g) of DM and neutral-detergent fibre, concentrations of NH_3–N (mg/l), lactate (mg/l) and total* VFA *(mmol/l), molar proportions (mol/100 mol) of individual* VFA *and acetate/propionate ratio (mol/mol) in sheep and in BC of rumen microorganisms inoculated with sheep ruminal fluid*

		Diet*					*P*-value		
Item†		HFA	HFG	HCA	HCG	S.E.M.	FCR	FOR	FCR × Forage
Apparent degradability									
DM	Sheep	0·51	0·49	0·42	0·44	0·088	<0·001	0·967	0·060
	BC	0·58	0·57	0·61	0·62	0·024	<0·001	0·449	0·079
NDF	Sheep	0·62	0·63	0·52	0·58	0·014	0·005	0·079	0·106
	BC	0·40	0·39	0·34	0·35	0·012	0·222	0·678	0·284
NH_3–N	Sheep	184	83·1	204	122	12·5	0·041	0·004	0·533
	BC	300	204	429	369	16·4	0·002	0·003	0·122
Lactate	Sheep	36	29	39	338	3·0	0·112	0·115	0·966
	BC	20	14	19	15	1·9	0·883	0·268	0·268
Total VFA	Sheep	108	84	98	90	2·9	0·668	0·001	0·117
	BC	61	53	73	63	2·2	0·010	0·013	0·816
Molar proportions of									
Acetate	Sheep	66·2	68·0	62·7	63·4	0·45	<0·001	0·136	0·112
	BC	63·4	64·3	57·5	58·9	0·65	<0·001	0·144	0·331
Propionate	Sheep	17·3	18·6	17·6	18·0	0·30	0·510	0·004	0·352
	BC	19·5	22·6	18·5	21·1	0·72	0·733	0·011	0·707
Butyrate	Sheep	11·9	10·5	15·2	14·9	0·40	<0·001	0·049	0·129
	BC	11·7	9·9	18·0	15·0	0·57	<0·001	0·010	0·859
~~Molar proportions of~~									
Isobutyrate	Sheep	1·27	0·79	1·20	0·84	0·076	0·631	0·003	0·156
	BC	1·13	0·57	1·34	0·97	0·105	0·144	0·006	0·324
Isovalerate	Sheep	1·19	0·70	1·22	0·94	0·032	0·020	<0·001	0·124
	BC	1·87	1·04	2·63	1·79	0·177	0·046	0·004	0·642
Valerate	Sheep	1·94	1·14	1·68	1·45	0·023	0·340	<0·001	<0·001
	BC	2·11	1·37	1·82	1·78	0·139	0·706	0·031	0·041
Caproate	Sheep	0·22	0·27	0·41	0·43	0·017	<0·001	0·200	0·250
	BC	0·21	0·16	0·24	0·29	0·030	0·046	0·950	0·335
Acetate/propionate	Sheep	3·85	3·67	3·56	3·52	0·076	0·397	0·082	0·133
	BC	3·25	2·85	3·11	2·79	0·117	0·262	0·029	0·413

* HFA: 700 g of alfalfa hay/kg DM; HFG: 700 g of grass hay/kg DM; HCA: 300 g alfalfa hay/kg DM; HCG: 300 g grass hay/kg DM. The same concentrate was used in all diets.

† Values for pH, NH_3–N, lactate and VFA in sheep are means across sampling times of 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 and 22 h after the morning feeding. Values in BC were measured after 24 h incubation.

lower ($P < 0·05$) in both fermentation systems for HF compared with HC diets, but no differences were observed in lactate concentrations.

The lack of FCR effect on VFA concentrations in sheep contrasts with the greater ($P = 0·010$) VFA concentrations observed for HC diets in the BC. There was good agreement between BC and sheep in the interpretation of the effects of FCR on VFA profile, and in both systems fermentation of HF diets resulted in greater ($P < 0·05$) proportions of acetate and acetate : propionate ratio, and lower ($P < 0·05$) proportions of butyrate, isovalerate and caproate compared with the fermentation of HC diets.

Both fermentation systems detected similar differences attributable to forage type for most parameters measured. Diets containing alfalfa hay promoted higher ($P < 0·05$) apparent DMD, NH_3–N and total VFA concentrations, and proportions of butyrate, isobutyrate, isovalerate and valerate than those containing grass hay in both sheep and BC. Degradability of NDF tended to be higher ($P = 0·079$) for alfalfa hay-diets than for grass hay-diets

Table 3. *Effect of FCR and type of forage on values of Shannon index and numbers of peaks detected in the ARISA electropherograms of samples from BC of rumen microorganisms and from the sheep rumen fluid (IN) used to inoculate the BC, and similarity index (%) of ARISA profiles between BC and IN*

	Diet*					*P*-value		
Item	HFA	HFG	HCA	HCG	S.E.M.	FCR	Forage	FCR × Forage
Shannon index								
BC	4·41	4·35	4·39	4·39	0·019	0·725	0·185	0·205
IN	4·51	4·42	4·46	4·44	0·019	0·631	0·030	0·105
Number of peaks								
BC	82·5	78·3	80·8	80·5	1·42	0·866	0·165	0·209
IN	90·5	83·0	86·5	85·3	1·53	0·593	0·030	0·108
Similarity index BC–IN	74·7	67·2	71·9	71·9	1·98	0·662	0·111	0·110

* HFA: 700 g of alfalfa hay/kg DM; HFG: 700 g of grass hay/kg DM; HCA: 300 g alfalfa hay/kg DM; HCG: 300 g grass hay/kg DM. The same concentrate was used in all diets.

in sheep, but no differences due to forage type were detected in BC.

There were differences between fermentation systems in the magnitude of most determined parameters. Values of pH were higher ($P < 0{\cdot}001$) in BC than in sheep (0·37 and 0·47 units for HF and HC diets, respectively). Sheep had apparent DMD values lower ($P < 0{\cdot}001$) than BC for both HF (0·502 and 0·574 g/g, respectively) and HC diets (0·471 and 0·617 g/g, respectively). For all the diets, values of NDFD were between 1·55 and 1·69 times lower in BC than in sheep. Concentrations of NH_3–N in BC were between 1·63 and 3·02 times higher than *in vivo*, whereas lactate and total VFA concentrations were 0·47–0·55 and 0·57–0·74 of those *in vivo*, respectively. Despite the differences in the magnitude of total VFA concentrations, there were significant relationships between *in vivo* values and those in BC ($n = 16$) for acetate ($r = 0{\cdot}831$; $P < 0{\cdot}001$), propionate ($r = 0{\cdot}647$; $P = 0{\cdot}007$) and butyrate ($r = 0{\cdot}582$; $P = 0{\cdot}018$) proportions and acetate : propionate ratio ($r = 0{\cdot}675$; $P = 0{\cdot}004$).

A total of 181 peaks were detected in the ARISA electropherograms across the full set of 32 samples, and the number of peaks in individual samples ranged from 75 to 94 in the inocula and from 65 to 90 in the BC samples. As shown in Table 3, the number of peaks and Shannon index in samples from BC were unaffected by FCR and forage type, but inocula from sheep fed diets with alfalfa hay had higher ($P = 0{\cdot}030$) number of peaks and Shannon index than inocula from grass hay-fed sheep. The similarity index between the bacterial communities in the inocula and those in the corresponding BC was unaffected by the studied dietary factors.

Figures 1(*a*) and (*b*) show the dendrograms of the ARISA profiles of inocula and BC samples for diets containing alfalfa hay and grass hay, respectively. No clear clustering according to fermentation system or FCR was observed, but for diets containing alfalfa hay samples from Sheep 4 and 3 formed two clearly differentiated clusters; similarly, samples from Sheep 2 clustered together in the dendrogram constructed for diets with grass hay.

DISCUSSION

The BC are frequently used to analyse rumen fermentation characteristics of different diets, and from a practical point of view it would be interesting to assess whether they can detect differences between diets similar to those observed *in vivo*. Therefore, the effects of FCR and forage type on rumen parameters in BC were assessed, and the results were compared with those obtained in the sheep used as rumen fluid donors.

The lack of marked differences in pH between HF and HC diets observed in BC was partly due to the high buffer capacity of the buffer–mineral solution, which prevented a pH drop in the BC with HC diets. Therefore, the pH decrease observed in sheep fed HC could not be reproduced in the BC, and this was also reflected in the greater *in vitro* pH values compared with those in sheep observed for all diets. Concentrations of NH_3–N in BC were higher than those *in vivo* and much higher than those usually

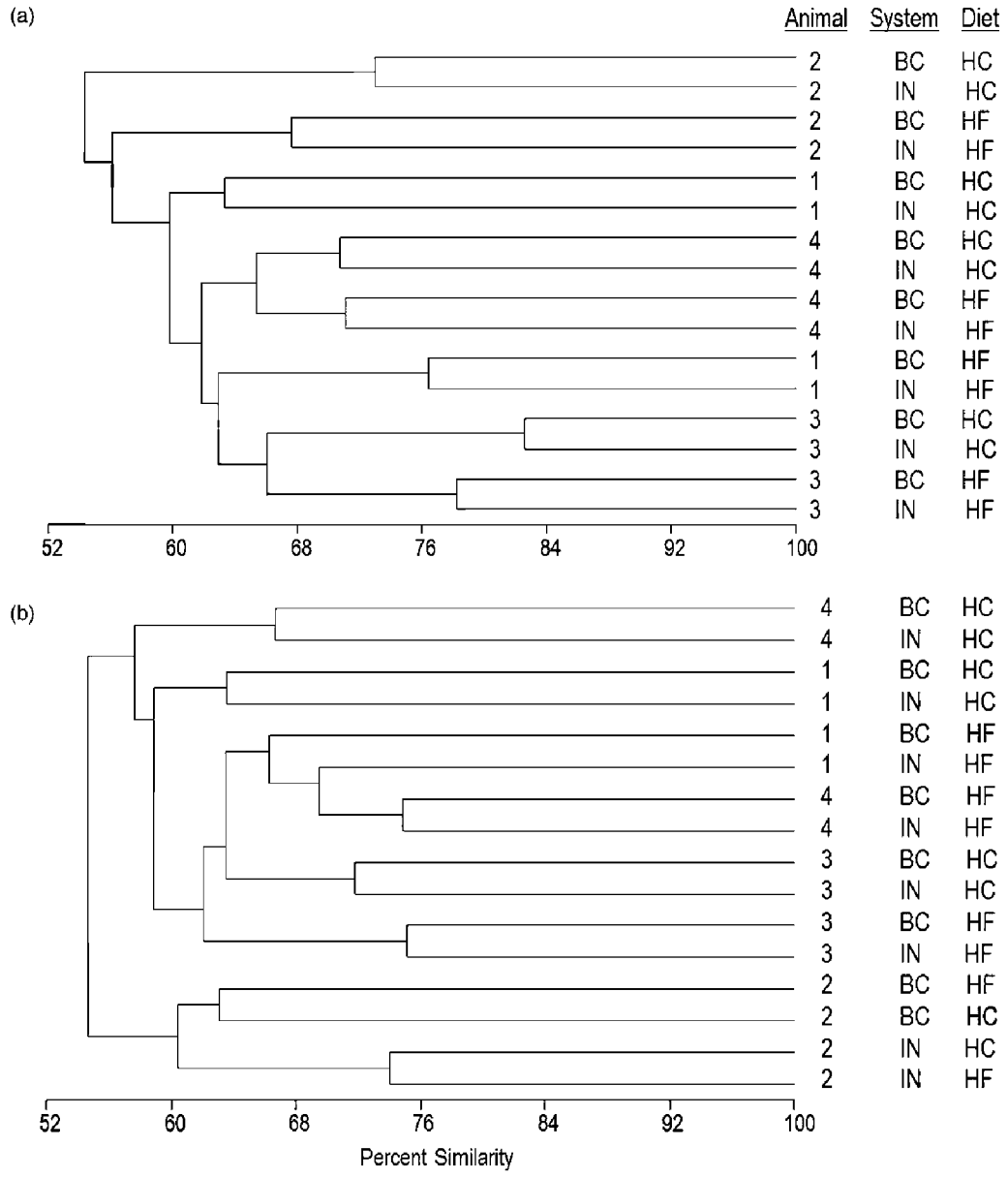

Fig. 1 - B/W online, B/W in print

Fig. 1. Dendrograms of ARISA profiles of bacterial communities in ruminal fluid from sheep used as IN and in BC after 24 h of incubation for diets with either 700 (HF) or 300 (HC) and alfalfa hay (Fig. 1(*a*)) or grass hay (Fig. 1(*b*)) as forage. Numbers 1–4 correspond to individual sheep. In each case, sheep were fed the same diet incubated in the BC.

observed in the rumen of animals fed similar diets (Mackie *et al.* 1978; Carro *et al.* 2000). This was possibly due to the use of an N-enriched buffer–mineral solution, but also to the lack of absorption in the BC. Mean values of NH_3–N concentrations in the inocula used to fill the BC were 131, 105, 167 and 167 for HFA, HFG, HCA and HCG diets, respectively. In addition, it should be taken into account that the modified buffer–mineral solution of Goering & Van Soest (1970) used in the present study supplied 84·11 mg N per litre in the form NH_4HCO_3, which can be rapidly degraded to NH_3 by ruminal microorganisms. These results would indicate that reducing the amount of N in the buffer–mineral solution (i.e., NH_4HCO_3 being replaced by $NaHCO_3$) would help to achieve more physiological NH_3–N concentrations in BC when incubating diets similar to those used in practical feeding of ruminants. In contrast, lactate concentrations were lower in BC than *in vivo*, which confirms previous studies (Tejido *et al.* 2005; Mateos *et al.* 2013). These results may suggest either lower lactate production or greater lactate utilization in BC

compared with sheep, but probably the high buffer capacity of the buffer–mineral solution prevented low pH values that might have stimulated the growth of lactate producers.

Absolute amounts of feed input and rumen digesta outflow are different in the *in vivo* and in the *in vitro* systems, and that could explain some of the observed differences in the magnitude of several parameters. The lower concentrations of total VFA in BC compared with the sheep rumen agree with previous observations (Rymer & Givens 2002; Brown *et al.* 2002) and were attributed to the lower feed/rumen fluid ratio in the BC (0·01; 0·4 g DM in 40 ml) than that *in vivo* (0·17; 1,184 g DM in an estimated rumen volume of 7000 ml; Ranilla *et al.* 1998). Moreover, dilution of ruminal fluid with the buffer–mineral solution before incubation also leads to a lower initial concentration of VFA in the BC. However, it should also be considered that VFA are removed from the rumen by absorption and flow to the lower tract, whereas there is no absorption or digesta flow in the BC. Despite the differences in the magnitude of total VFA concentrations, significant relationships between both measurements were observed for the main VFA proportions. This is in agreement with results of Rymer & Givens (2002), who also found significant relationships between the VFA pattern in BC and in the rumen of sheep fed three mixed diets. In contrast, Brown *et al.* (2002) reported no relationship between the VFA measured in BC and those in the rumen of steers fed eight different forages; they attributed the lack of relationship to the use as IN for the BC trial of rumen fluid from a different animal species (sheep). In addition, the *in vivo* experiment was conducted with steers fed only forage, whereas the sheep donors of rumen fluid for the *in vitro* experiment were fed a mixed diet (600 g forage/kg and 400 g concentrate/kg).

The significant relationships observed in the current study between *in vivo* and *in vitro* VFA profile would indicate a similar fermentation pattern in both systems, although in paired *t* test comparisons acetate proportions were lower ($P < 0·001$) and those of propionate greater ($P = 0·002$) in BC than in sheep. As pointed out by Rymer & Givens (2002), some differences between *in vitro* and *in vivo* fermentations can be partly explained by fibrolytic bacteria being more active *in vivo v. in vitro*. Manipulation of rumen contents for its use as IN inevitable implies a certain exposure to oxygen that reduces the viability of the fibrolytic bacteria (Russell & Wilson 1996). This would help to explain the lower acetate proportions in the BC, which is consistent with the lower ($P < 0·001$) NDFD observed in the *in vitro* system, as acetate is mainly produced from the fermentation of structural carbohydrates. The higher concentrations of isovalerate ($P < 0·001$) and valerate ($P = 0·049$) in the BC than in sheep may also indicate a lower capture of these branched-chain VFA by fibrolytic bacteria, as they use these VFA for the synthesis of essential amino acids, long-chain fatty acids and aldehydes (Bryant 1973).

The similarity index between the bacterial communities in the ruminal fluid from sheep used as IN and those in the corresponding BC ranged from 67·2 to 74·7% for the different diets. These values are similar to the 70·2% of similarity found by Prates *et al.* (2010) when comparing the bacterial communities' structure in semi-continuous BC with alfalfa hay as substrate after 24 h of incubation with that in the rumen fluid used as IN. These results would indicate that some changes in bacterial communities were produced over the incubation period. Different conditions in BC, such as the buffer capacity of the incubation medium, feeding rate (substrate/cultivation medium), digesta retention time, movements, etc., compared with the sheep rumen may have caused a selection of some bacterial strains. Given the wide range of bacteria inhabiting the rumen, it would be expected that some community members adapt well to the environmental conditions in BC, whereas others cannot grow under the same conditions. In accordance with this hypothesis, in paired Student's *t* test comparisons across diets both the number of peaks and Shannon index' values were lower ($P < 0·001$) in BC (79·3 and 4·37, respectively) than in the inocula (86·3 and 4·46), and 14 peaks appearing in the inocula samples were never detected in any BC sample. When individual pairs of samples (inocula for each diet and sheep and its corresponding BC) were compared with the number of peaks appearing in the inocula and not detected in BC ranged from 21 to 34, and this number was not affected ($P > 0·05$) by FCR (mean values of 28·8 and 26·5 for HF and HC diets, respectively), forage type (mean values of 26·6 and 28·6 for diets containing alfalfa and grass hay, respectively) or individual sheep (28·0, 26·5, 27·5 and 28·5 for sheep 1, 2, 3 and 4, respectively). These results seem to indicate that environmental conditions in BC were the main factors influencing transition of the bacterial communities, as diet characteristics and animal donor had no influence on the

Q2

number of peaks in the inocula not appearing in the BC.

The cluster pattern observed in the dendrograms would indicate that sheep had a larger effect on bacterial communities than diet or fermentation system. Interestingly, in all the cases each BC sample showed the highest similarity index with its corresponding IN, with the exception of samples from Sheep 2 fed the diet containing grass hay. These results highlight the importance of using an IN from donors fed a diet similar to that being incubated in BC when conducting *in vitro* experiments.

In conclusion, although there were differences between sheep and BC in the magnitude of most parameters, differences between diets attributed to forage type in BC were similar to those in sheep for most of the measured parameters. However, there were discrepancies between *in vivo* and BC in the interpretation of the effects of FCR on some parameters, such as pH, NDFD and total VFA concentrations. The similarity in bacterial communities between the IN and BC was >67% for all diets, but the lower bacterial diversity in BC compared with rumen fluid from sheep used as IN would indicate a selection of some bacterial strains over the incubation period. Studies identifying changes in specific microbial populations through the incubation period in BC would be helpful to understand the observed differences in fermentation parameters between *in vivo* and *in vitro* systems and might contribute to improve *in vitro* fermentation systems. Moreover, reducing the NH_4HCO_3 concentration in the buffer-mineral solution would help to achieve more physiological NH_3–N concentrations in BC when incubating diets similar to those used in practical feeding of ruminants.

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